Fig. 4. Part of the cephalic lobe (A) and caudal lobe (B). 1, carminophilic acidophils arranging in cords around sinusoid; 2, amphophils; 3, orangeophilic acidophils of small size; 4, basophils. Azan stain. A–B, ×460.

the turtle [11–13].

The portal vessels break up into secondary capillary plexus in the PD. In the present studies, the capillaries deriving from the anterior group of portal vessels mainly supply blood into the cephalic lobe of the PD, whereas the capillary plexus deriving from the posterior group of portal vessels supply blood into the caudal lobe of the PD. This arrangement of the vascular supply is also in good accord with previous reports [4]. It should be noted that the portal vessels and secondary capillary net in the turtle, *Chrysemys picta* are not separated [2].

It has been clearly demonstrated that there is distinct cytological differentiation between the cephalic and caudal lobes of the turtle PD. Early investigations based on tinctorial studies have shown five chromophilic cell types, consisting of two types of acidophils, and three basophils in the turtle PD [8, 9, 14, 15]. The close relationship between the chromophilic cell types and the specific hormone secretions has been studied by using immunocytochemical techniques [16, 17]. Recently, Mikami [18] has identified five types of secretory cells in the turtle PD (*Geoclemys reevesii*): cephalic lobe (carminophilic acidophils as prolactin-immunoreactive cells, amphophils as adreno-

corticotropic hormone-immunoreactive cells), and caudal lobe (orangeophilic acidophils as growth hormone-immunoreactive cells, two types of basophils as gonadotropin- and thyrotropin-immunoreactive cells, respectively).

In the previous study of the vascularization of the turtle, *Pseudemys scripta*, we raised the question that the presence of two distinct groups of capillary plexus and portal vessel system might be correlated with the cytological differentiation of the PD. The present study has clearly showed that the regional specialization of two distinct groups of portal vessels is correlated with the cytological differentiation of the PD in the turtle, *Geoclemys reevesii*. It may be also postulated that the anterior region of the ME controls the cephalic lobe and the posterior region of the ME controls the caudal lobe of the PD. Similar structural differentiation of the vascular system of the hypophysis has been demonstrated in the bird and the functional relationship between ME and PD has been suggested [19–21].

Few references correlating immunocytochemical studies can be found for the hypophysiotropic factors of reptiles. There is strong evidence that the release of luteinizing hormone from the PD is controlled by luteinizing hormone releasing hor-

mone (LHRH). The occurrence of LHRH-nerve terminals has been demonstrated in the ME of various reptiles including the turtle, *Geoclemys reevesii* [22]. In the lizard, neurons containing immunoreactive somatostatin or β-endorphin have been detected in the ME [23, 24]. The presence of the fibers containing the hypophysiotropic factors in the ME may be involved in the control of the PD function by portal vascular system.

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Fetal and Postnatal Development of Arginine Vasopressin-Immunoreactive Neurons in the Mouse

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ABSTRACT—The ontogeny of arginine vasopressin (AVP)-producing neurons in the hypothalamo-neurohypophysial system (HNS) was immunocytochemically studied in fetal and postnatal mice. Presumptive AVP neurons underwent proliferation in the vicinity of the third ventricle and migrated to settle in the final loci by fetal age of 18 days (FA 18). AVP-immunoreactive neurons were first detected on FA 14 in the presumptive supraoptic (SON) and retrochiasmatic nuclei (RCN). AVP-immunoreactive axons and terminals were present in the median eminence and pars nervosa of the neurohypophysis on FA 15, but not on FA 14. Immunoreactive neurons were recognized in the paraventricular nucleus (PVN) on FA 15 and their terminals in the external layer of the median eminence became immunoreactive on FA 18. In the suprachiasmatic nucleus (SCN) AVP-immunoreactive perikarya appeared on FA 16. The number of AVP neurons in the SON and RCN markedly increased during fetal life. Postnatal increase in the number of immunoreactive neurons in the PVN and SCN as well as that of the SON and RCN was apparent. To sum up, the present study shows that cytodifferentiation of AVP-producing neurons in the HNS takes place during early days of last trimester of pregnancy and that the HNS completes the general morphological changes before birth.

INTRODUCTION

Arginine vasopressin (AVP) is synthesized mainly in the magnocellular neurons in the supraoptic (SON), retrochiasmatic (RCN) and paraventricular nuclei (PVN) of the hypothalamus in mammals [1–5]. AVP is transported through the fiber layer of the median eminence (ME) to the pars nervosa (PN) of the pituitary and to the external layer of the ME [6]. Some other AVP-producing neurons are present among the parvocellular neurons in the suprachiasmatic nucleus (SCN), the axons originating from which projecting to the brain regions other than the PN and ME [7, 8]. Besides the vasopressinergic pathways from the PVN to the PN and ME [9–12], extrahypothalamic projections have also been documented, for example, to the forebrain [8, 12], the brain stem [8, 13] and the spinal cord [8, 13, 14]. Therefore, AVP has been proposed to act not only as the antidiuretic hormone but also as a neurotransmitter.

During the last ten years the ontogeny of the hypothalamo-neurohypophysial system (HNS) has been investigated in some species of rodents. Histological staining [15], electron microscopy [16–20], immunocytochemistry [21–23] and radioimmunoassay [19, 24, 25] were applied for the study of development of the HNS. These studies have demonstrated that AVP synthesis in the magnocellular neurons begins to occur and rapidly elevates during the late gestation period and that the HNS completes its maturation by the end of the first month of life. Compared to the studies on other species of mammals, however, developmental study on the mouse HNS is scanty.

Therefore, in the present study the development of AVP-producing neurons of the HNS in fetal and postnatal mice was examined by means of AVP immunocytochemistry.

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* Fetal ages in the cited paper were rearranged for convenience of comparison with the present study, so that the day when vaginal plug was observed was designated as FA 0.
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MATERIALS AND METHODS

Animals

Mice of the C57BL/6NCrj strain maintained in this laboratory were used in the present study. They were housed in a temperature-controlled room at 12-hr light (06:00–18:00 hr) and 12-hr dark cycle with free access to laboratory chow (CA–1, Japan Clea Inc.) and tap water.

Female mice were placed in a cage with males in the evening and separated from males in the next morning. The day was designated as fetal age of 0 day (FA 0) for successful pregnancy. Most pregnant mothers delivered their pups in the morning on FA 19. The day of birth was designated as postnatal age of 0 day (PA 0).

Tissue preparation

Fetuses between FA 14 and FA 18 regardless of sexes were taken out by Caesarean cut at 13:00 hr from at least two different litters at each fetal age. After birth, in order to exclude any possible sex difference, only male mice were chosen and killed at 13:00 hr on PA 2, 14, 30 and 90. Five animals were used for each fetal and postnatal age groups.

Animals were killed by decapitation, and the brains were taken out and fixed in Bouin's fluid for two days. After trimming all the brains were kept in 70% ethanol overnight. Dehydration in a graded series of ethanol and embedding in paraplast were completed within the following day. Serial frontal sections were cut at 6 μm in thickness, and every fifth sections were mounted on albumin-coated glass slides for immunocytochemical staining. The adjacent sections were stained with Ehrlich's haematoxyline-eosin (fetuses) or thionine (postnatal mice) for general histological changes during development of the HNS.

Immunocytochemical procedures

Immunocytochemistry for AVP was performed by means of the avidin-biotin-peroxidase complex (ABC) technique [26]. Deparaffinized sections were reacted with the following sequence of solutions: (1) rabbit anti-AVP serum (RV-1K, raised in this laboratory) (1:6,400) for 24 hr at 4°C, (2) biotinylated goat anti-rabbit IgG serum (Vector

Laboratories, California) (1:200) for 30 min at room temperature (RT), (3) 0.3% H_2O_2 for 30 min at RT, (4) ABC reagents (avidin DH and biotinylated horseradish peroxidase H) of the ABC kit (Vector Laboratories, California) (1:100) for 30 min at RT and (5) 0.015% 3,3'-diaminobenzidine in 0.05 M Tris-HCl buffer (pH 7.4) containing 0.01% H_2O_2 for 10 min at RT. Sections were washed three times with 0.01 M phosphate buffered saline (pH 7.4) at 4°C, 5 min each, between each step. The characterization of anti-AVP serum was reported previously [27].

After washing in three changes of distilled water, the preparations were dehydrated with graded series of ethanol and mounted with balsam.

Morphometry

The right halves of the hypothalamus were used for morphometry. In immunocytochemically stained sections, only those cells that showed distinct nucleoli and contained brown reaction products were counted as AVP-producing neurons. The total number of cells (N) per animal was calculated from the total number of cells (n) in every fifth sections by the formula: $N = 2 \times 5 \times n$.

RESULTS

1. Development of the HNS

FA 14. General morphology of the hypothalamus was extremely different from that in the adults (Fig. 1a). Presumptive magnocellular neurons appeared to be produced in the diamond-shaped region around the third ventricle at the junction of the ventral and medial lobes of the diencephalic neuroepithelium. Ependymal cells were spindle-like in shape, forming two or three layers.

The condensation of the SON, RCN and PVN was not completed on FA 14. However, in the presumptive region of the SON, the magnocellular neurons were a little more concentrated than the surrounding area. The presumptive PVN contained the cells migrating from the neuroepithelium to the final locus in the hypothalamus. The SCN was not yet demarcated from the adjacent area. The ependymal cells along the ventral floor of the third ventricle were still undergoing mitosis.
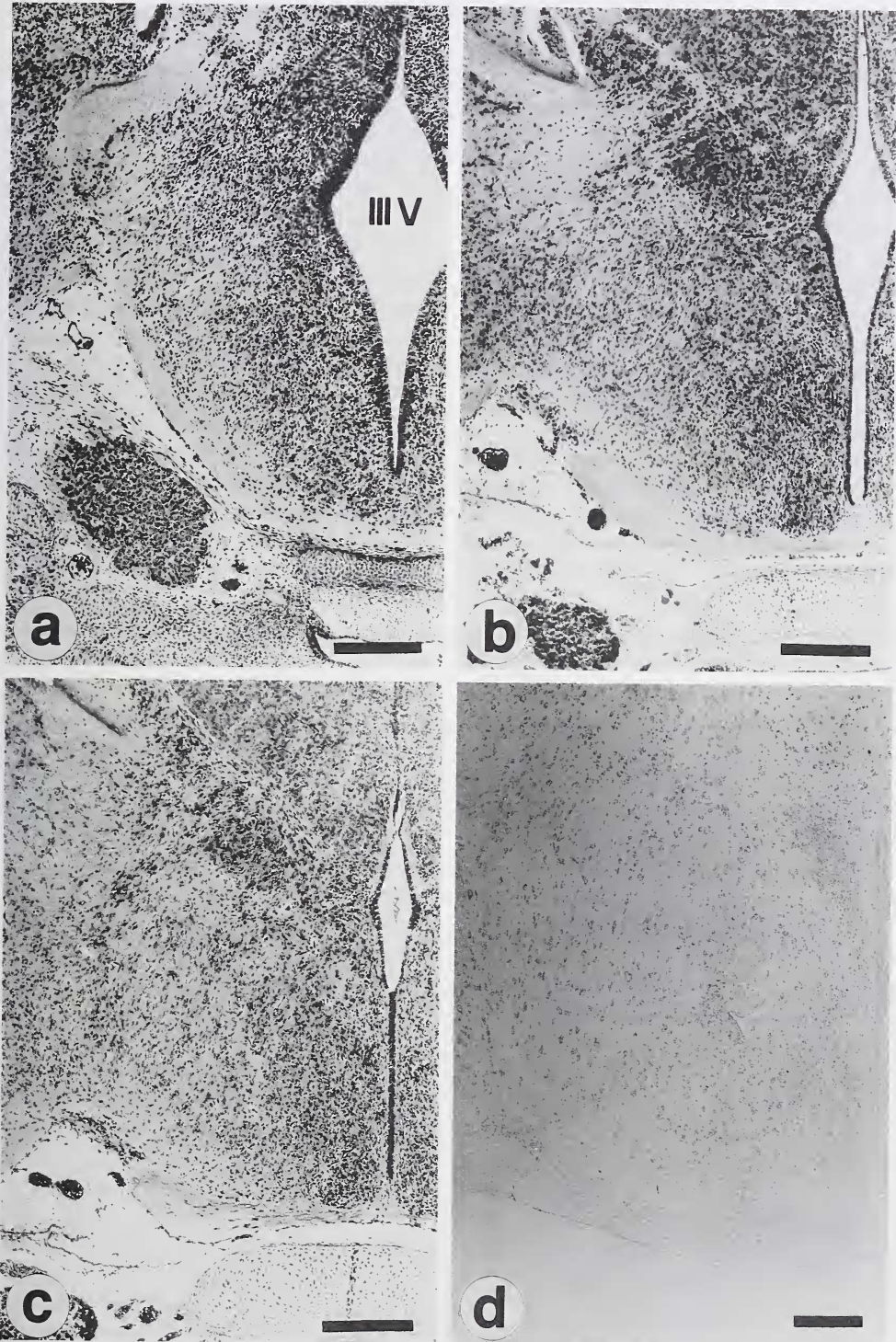
Fig. 1. Frontal sections of the hypothalamus (right side) during development. Stained with haematoxyline and eosin (a-c), or thionine (d). a, FA 14; b, FA 16; c, FA 18; d, PA 14. IIIV, third ventricle. Bars: 200 μm.

The ME was consisted of the ependymal and fiber layers, and the external layer was not yet differentiated. Scattered cells of the pars tuberalis were visible under the ME. The PN was immature and full of glial cells (primordial pituicytes) surrounding a central cavity and possesed very few, if any, axonal termini. The periphery of the PN was well vascularized.

FA 15. Ependymal cells in the diamond-shaped region around the third ventricle continued to divide, and some of them might differentiate into the hypothalamic cells, because the migratory paths of cells from the matrix layer along the third ventricle to the ventro-lateral region of the hypothalamus was observed.

At this fetal age the SON could be recognized as the cell cluster laterally to the optic tract, but many neurons were still migrating to the SON. The PVN appeared to contain both migrating and settled cells. The RCN and SCN were not clearly demarcated.

The fiber layer of the ME was better developed than that on FA 14. Though the external layer of the ME was not at all differentiated, the number of cells of the pars tuberalis was increased on the ventral surface of the ME. Because of the penetration of axons to the outer area of the PN, it was clearly divided into two areas; the inner pituicyte-rich area and the outer fibrous area.

FA 16. By this day, most ependymal cells lining the third ventricle appeared to cease any further mitotic divisions, and the neurons migrating to the ventrolateral regions of the hypothalamus were very few (Fig. 1b). Morphogenesis of the SON was almost completed on FA 16, but a few neurons were still migrating to this nucleus. The RCN and PVN were clearly recognized as cell clusters. The SCN showed itself as a slightly dense area on the optic chiasma.

General morphology of the neurohypophysis (ME and PN) did not show much difference as compared to that on FA 15. The penetration of blood vessels into the pars tuberalis and the inner area of the PN was the characteristic phenomenon at this stage.

FA 17. The cytoplasm of magnocellular neurons in the SON and PVN was not well-developed. The parvocellular SCN formed a dis-crete structure, locating as a pair close to the medial optic chiasma.

The external layer of the ME was developed. The PN was markedly developed on FA 17, and in some fetuses the central cavity had disappeared. Pituicytes dispersed all around the PN and inter-mingled with axons, so that exclusively fibrous area was left only at the periphery.

FA 18. On this last day of embryonic life, the hypothalamic nuclei containing AVP-producing neurons completed development (Fig. 1c). The adult-type partition of the RCN into lateral and medial groups was observed.

Further development of the external layer of the ME was seen, but the layer was thinner than that in adult animals. Pituicytes further dispersed in the PN, and among these cells a greater number of axons was present than on FA 17.

These observations indicate that the mor-phogenesis of the HNS is almost completed prior to the delivery.

Postnatal development. The postnatal development of the hypothalamus was characterized by the hypertrophy of each neuron (Fig. 1d). The nucleoli in the neuronal nuclei became distinct. Though the hypothalamic neurons were still undergoing condensation on PA 2, the intercellular spaces became spread after PA 14. However, the hypothalamic nuclei were obviously distinguished from the surrounding area during the postnatal maturation.

After birth, the development of the fiber and external layers of the ME further advanced, and the growth of the PN was apparent.

2. Development of AVP-producing Neurons

The changes of AVP immunoreactivity of the hypothalamus and neurohypophysis during fetal and postnatal development are summarized in Table 1.

Fetal life

Development of the hypothalamus. On FA 14, some neurons of the presumptive SON in three out of the five fetuses were weakly reactive to anti-AVP serum. Weak AVP immunoreactivity was also detected in the ventro-caudal part of the RCN in all animals. The number of immunoreactive